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Role of DNA-Dependent RNA Polymerases II and III in Transcription of the Adenovirus Genome Late in Productive Infection

(RNA polymerase/α-amanitin/5.5S viral RNA/5S host RNA)

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ABSTRACT **DNA-dependent RNA polymerases I, II, and III were isolated and partially purified from KB (human) cells 18 hr after infection with adenovirus 2. As reported previously for the enzymes from other animal cells, RNA polymerase II was completely sensitive to low concentrations of α-amanitin (50% inhibition at 0.02 μg/ml), RNA polymerase III was completely sensitive to high concentrations of α-amanitin (50% inhibition at 20 μg/ml) and RNA polymerase I was totally resistant to concentrations of α-amanitin less than or equal to 200 μg/ml. RNA synthesis by the endogenous RNA polymerase activities in nuclei isolated from infected cells was completely sensitive to α-amanitin, thus suggesting that RNA polymerase I is not involved in viral DNA transcription even though it is present in these cells. The α-amanitin inhibition curve was biphasic and showed inflection points at about 0.02 and 20 μg/ml, suggesting the participation of both RNA polymerases II and III in the synthesis of RNA in these nuclei. Furthermore, at least a large fraction of the synthesis of the nuclear precursors to viral mRNA, monitored by hybridization to viral DNA, showed the same sensitivity to α-amanitin as did RNA polymerase II; and the synthesis of both viral 5.5S RNA and (presumably cellular) 5S RNA in the isolated nuclei exhibited the same sensitivity to α-amanitin as did purified RNA polymerase III. Thus, these data provide strong supporting evidence for previous studies which suggested the involvement of an RNA polymerase II in transcription of the adenovirus genome and demonstrate the role of an RNA polymerase III activity in the synthesis of viral 5.5S RNA and cellular 5S RNA.**

Cultured human (KB) cells infected with adenovirus 2 provide an attractive model system for analysis of RNA metabolism in eukaryotic cells, since many features of viral RNA synthesis, processing and transport appear to be similar to the respective cellular processes (1, 2). Furthermore, transcription of the adenovirus genome during the lytic cycle is regulated. During the early phase of productive infection, prior to the onset of viral DNA synthesis, only a limited portion of the viral genome is transcribed, while later in infection a large fraction of the remainder of the genome is transcribed (3–5). Several features of the adenovirus system make it advantageous for investigating the regulation of transcription. Analysis of the products of transcription is facilitated by the small size (6) and availability in large amounts of intact viral genomes. Moreover, unique segments of the adenovirus genome can be generated by restriction endonucleases and used to localize specific transcripts to defined portions of the genome (7). In addition, since many viral genomes are simultaneously transcribed in each cell late in the lytic cycle, the components involved in regulating the transcription of specific genes may be more readily identified in this system than in those where the cellular concentrations of specific activated genes are much lower.

One approach to a further analysis of the regulation of viral gene expression is to identify the RNA polymerases involved in transcription of the viral genome and study their specific functions and the regulation of their activities. Three major classes of nuclear RNA polymerases have been defined in animal cells (8). The nucleolar RNA polymerase I (9) synthesizes rRNA (10–12) and is resistant to high concentrations of α-amanitin (13, 14). The nucleoplasmic RNA polymerase II is sensitive to low concentrations of α-amanitin (15, 16) and is probably involved in transcription of the nuclear precursors to mRNA (10–12). The nucleoplasmic RNA polymerase III is sensitive to high concentrations of α-amanitin (13, 14) and is responsible for the synthesis of the 5S RNA component of ribosomes and of tRNA precursors (14).

Previous studies with nuclei isolated from infected cells suggested that most of the adenovirus genome is transcribed by a form of RNA polymerase II, for most viral RNA synthesis was sensitive to low concentrations of α-amanitin (17, 18). The adenovirus genome codes for a low-molecular-weight 5.5S RNA which is synthesized in large amounts but whose function is unknown (19). Synthesis of 5.5S RNA in isolated nuclei was resistant to low α-amanitin concentrations, suggesting the involvement of an enzyme distinct from RNA polymerase II (20).

The objective of the present study was to further define the role of cellular RNA polymerases (or modified forms thereof) in the synthesis of viral RNAs transcribed late in productive infection (18 hr). Utilizing the unique α-amanitin sensitivities of the cellular RNA polymerases, we show here that a class III RNA polymerase activity is responsible for the synthesis of 5.5S RNA, that the majority of nuclear adenovirus RNA is indeed synthesized by a type II RNA polymerase, and that RNA polymerase I is apparently not involved in adenovirus transcription late in infection.

MATERIALS AND METHODS

Uninfected and infected KB cells were grown in suspension as described (21). Nuclei were prepared by the method of Price and Penman (17) without the use of detergents. Conditions for incubation and procedures for RNA extraction and

Abbreviations: 4S RNA, low-molecular-weight fraction containing the tRNAs; 5S RNA, low-molecular-weight ribosome-associated RNA; 5.5S RNA, viral-coded small-molecular-weight RNA.

electrophoresis on polyacrylamide gels are described in the appropriate *figure legends*. Nitrocellulose filters containing adenovirus DNA were prepared and hybridization reactions were carried out under conditions previously described (22). Viral 5.5S [^{32}P]RNA and unlabeled viral 5.5S RNA were purified as described (19, 23).

RESULTS

Late in productive infection (18 hr) most of the DNA, RNA, and protein synthesis in these cells is virus specific. At least 50% of the newly synthesized nuclear RNA (M. Brunner and H. J. Raskas, in preparation) and 80% of pulse labeled polysomal mRNA (24, 25) hybridize specifically to viral DNA. In order to define the enzymes participating in transcription of the viral genome *in vivo*, the first step was to isolate, identify, and characterize the RNA polymerases present in cultures of human KB cells 18 hr after infection. When solubilized RNA polymerase extracts (9) were subjected to DEAE-Sephadex chromatography, three peaks of activity corresponding to RNA polymerases I, II and III (8–11, 26, 27) were identified (28).

Using exogenous DNA as a template, we tested the three partially purified RNA polymerases for sensitivity to α-amanitin. As shown in Fig. 1, RNA polymerase II is inhibited by low concentrations of α-amanitin (50% inhibition at 0.018 μg/ml), RNA polymerase III is inhibited at 1000-fold higher concentrations (50% inhibition at 19 μg/ml), and RNA polymerase I remains unaffected at concentrations up to at least 200 μg/ml. Similar results were previously found for RNA polymerases isolated from mouse plasmacytoma cells (13, 14) and from *Xenopus laevis* oocytes and somatic cells (ref. 27 and R. G. Roeder, in preparation).

In order to relate the activities of the three RNA polymerases to the transcriptional events occurring *in vivo*, total endogenous RNA synthesis was studied in isolated nuclei prepared 18 hr after infection. As previously reported (17–20), such nuclear preparations are very active in the synthesis of RNA via endogenous templates and RNA polymerases. At an ionic strength (0.02 M ammonium sulfate) optimal for endogenous RNA polymerase III (28) a large fraction of the nuclear activity (about 60%) is sensitive to low concentrations of α-amanitin, while the remainder is inhibited by high concentrations of the toxin (Fig. 1). The inhibition curve is biphasic, with inflection points at α-amanitin concentrations similar to those necessary to inhibit purified RNA polymerases II and III by 50%, strongly suggesting that both of these two enzymes are active in transcription in infected cells. The endogenous activity in late infected KB cell nuclei was completely inhibited by high α-amanitin concentrations (Fig. 1), thus suggesting that RNA polymerase I is not active in these nuclei. Under similar incubation conditions, a major fraction of the endogenous RNA polymerase activity from uninfected KB cells (unpublished observations) and all of the endogenous activity in isolated nucleoli (14) are unaffected by high α-amanitin concentrations, thus ruling out nonspecific effects of the toxin in these endogenous systems.

The salt optima for the endogenous RNA polymerase activities (28) differ markedly from the optima reported (8, 13, 27) for the purified enzymes on purified templates (especially RNA polymerase III with optima at 0.06 M and 0.17 M ammonium sulfate), thus precluding correlations of

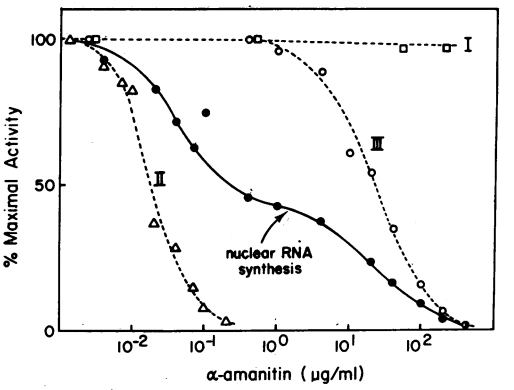

FIG. 1. Effect of α-amanitin concentration on purified RNA polymerases and on endogenous RNA polymerase activity in isolated nuclei. RNA polymerases were solubilized from 2 g of KB cells 18 hr after adenovirus infection and separated on a DEAE-Sephadex column as described (13). RNA polymerase III was further concentrated on phosphocellulose. This step has been shown not to affect the α-amanitin sensitivity of *Xenopus laevis* liver, ovary, embryo, or cultured kidney cell RNA polymerase III (R. G. Roeder, unpublished observations). Incubations were carried out at 0.06 M ammonium sulfate, using calf-thymus DNA template (120 μg/ml) and with 1 μCi of [^{3}H]UTP (0.5 Ci/mmol) per reaction, as described (27). The final α-amanitin concentration (premixed with substrate and template at 0° prior to enzyme addition) was varied as indicated. Reactions were carried out for 20 min at 37° and processed as described (27). The 100% values represent 12.7 pmol UMP incorporated for RNA polymerase I (□), 10.4 pmol of UMP incorporated for RNA polymerase II (△) and 12.5 pmol of UMP incorporated for RNA polymerase III (○). Nuclei from infected cells were assayed as described (10, 14), in the presence of 0.02 M ammonium sulfate, and with 1 μCi of [^{3}H]UTP per 25-μl reaction (0.7 Ci/mmole). Incubations were for 20 min at 37°. The 100% values for nuclear RNA synthesis (●) represent the incorporation of 4000 cpm into RNA per reaction.

specific enzyme functions with the synthesis of specific RNAs solely on the basis of ionic strength effects.

Therefore, to define unambiguously the functions of RNA polymerases II and III in virus-infected cells, the α-amanitin sensitivities of the syntheses of specific viral and cellular products in isolated nuclei were compared to the toxin sensitivities of the purified enzymes. Nuclear viral RNA, which is presumably the precursor to cytoplasmic mRNA (29), was isolated and identified by hybridization; small-molecular-weight RNA products were characterized by acrylamide gel electrophoresis. For the latter experiments ^{32}P-labeled marker RNAs were isolated from the cytoplasm of cells late in infection and electrophoresed with the *in vitro* RNAs. These markers included viral 5.5S RNA, the two conformational isomers of cellular 5S RNA (30, 31), and a group of 4S RNAs. Fig. 2*A* shows that nuclei from uninfected KB cells synthesize an RNA species corresponding, after denaturation, to one of the conformational isomers of 5S RNA, and a heterogeneous class of low-molecular-weight RNAs which presumably are precursors to 4S RNA (14, 20), as reported previously in other cell types (14, 20).

Nuclei from infected cells continue to synthesize the same low-molecular-weight RNAs synthesized in uninfected cells including a 5S RNA and a heterogeneous class of RNAs which are presumably tRNA precursors (Fig. 2*D–F*). However, the predominant low-molecular-weight RNA synthesized in nuclei from infected KB cells is an RNA species with

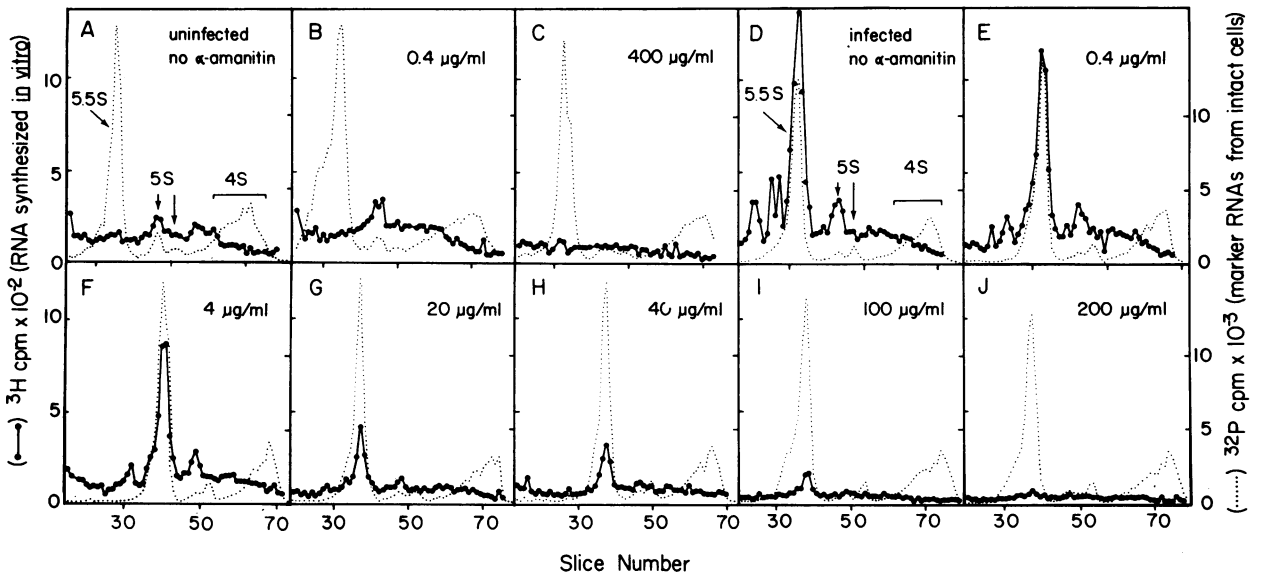

FIG. 2. Analysis of low-molecular-weight RNAs synthesized *in vitro* from uninfected (A-C) and adenovirus-infected (D-J) KB cells with increasing concentrations of α-amanitin. Nuclei were incubated as described in the *legend* to Fig. 1, except that 100 μCi (36.8 Ci/mmol) or 50 μCi (14 Ci/mmol) of [^{3}H]UTP per 50-μl reaction were used, respectively, for uninfected or infected cell nuclei. Incubations were for 10 min at 20°, in the presence of 0.02 M ammonium sulfate. RNA was extracted and denatured in 100% formamide as described (14). After dilution of the formamide to 50% with 1 M sucrose and addition of marker [^{32}P]RNAs synthesized *in vivo*, the RNAs were run on polyacrylamide gel slabs (12 × 15 × 0.1 cm) at 40 mA and 100 V for 5 hr (14). Strips of the slab gels were sliced, solubilized, and radioactivity measured as described (14). (···) marker [^{32}P]RNAs; (●) [^{3}H]RNAs synthesized by nuclei. The large [^{32}P]RNA peak (starting from the *left*) corresponds to 5.5S RNA (as determined from independent experiments with purified 7S and 5S RNA), the second double peak corresponds to two conformational isomers (30, 31) of native 5S RNA, and the broader [^{32}P]RNA peak on the *right* corresponds to 4S RNA. All values were corrected for spillover of ^{32}P into the ^{3}H channel (0.8%), but backgrounds were not subtracted.

the same electrophoretic mobility as the viral 5.5S RNA synthesized *in vivo* (Fig. 2*D* and ref. 19). The sequence identity of the 5.5S synthesized in nuclei with the 5.5S RNA synthesized *in vivo* has been demonstrated in the present study by hybridization competition. As shown in Table 1 a small percentage of the ^{3}H-labeled 5.5S RNA (*in vitro*) hybridizes to adenovirus DNA and this hybridization can be blocked by the addition of unlabeled 5.5S RNA synthesized *in vivo*. 5.5S [^{32}P]RNA from intact cells was included in these hybridization reactions as an internal standard (10). The efficiencies of hybridization for the 5.5S [^{3}H]RNA and for the 5.5S [^{32}P]RNA are similar. As observed for 5.5S [^{3}H]RNA, competition of 5.5S [^{32}P]RNA by unlabeled 5.5S RNA is significant but incomplete, probably because of nonsaturating levels of competitor. After correction for the incomplete competition observed, it appears that about 80% of the 5.5S [^{3}H]RNA synthesized in nuclei has nucleotide sequences indistinguishable from those in the 5.5S RNA from intact cells. Low hybridization levels occur presumably because only a small fraction of the genome codes for this RNA (30, 32) and because isolated nuclei may contain endogenous pools of unlabeled 5.5S RNA.

TABLE 1. *Hybridization to adenovirus DNA of 5.5S RNA synthesized by isolated nuclei or by intact cells*

Input RNA	cpm input	RNA hybridized, cpm −competitor	RNA hybridized, cpm +competitor
5.5S [^{32}P]RNA synthesized by intact cells	41,500	368	36
5.5S [^{3}H]RNA synthesized by isolated nuclei in the presence of 0.4 μg/ml of α-amanitin	9,000	125	37

5.5S RNAs synthesized either in intact cells (^{32}P) or in isolated nuclei (^{3}H) were hybridized with nitrocellulose filters containing 5 μg of adenovirus DNA as described in Fig. 3. Unlabeled competitor 5.5S RNA was used at a concentration of 100 μg/ml (10 μg per hybridization reaction mixture). 5.5S [^{32}P]RNA was included in the same hybridization mixture and served as an internal standard for the efficiency of hybridization and competition. All counts were corrected for spillover of ^{32}P into the ^{3}H channel and for background, which was 12 cpm for ^{3}H and 7 cpm for ^{32}P.

The α-amanitin sensitivities of the syntheses of the low-molecular-weight viral and cellular RNAs were examined next. As reported previously (20), low concentrations of α-amanitin (0.4 μg/ml) do not affect the amounts of viral 5.5S RNA (Fig. 2*D* and *E*) or host 5S RNA and 4S RNA precursor synthesized either in uninfected (Fig. 2*A* and *B*) or in infected cells (Fig. 2*D* and *E*). However, at higher α-amanitin concentrations, the rates of synthesis of all these low-molecular-weight components decrease in parallel, both in nuclei from uninfected cells (Fig. 2 *A–C*) or from infected cells (Fig. 2*D–J*).

The relative amounts of viral 5.5S RNA and cellular 5S RNA synthesized in nuclei isolated from infected cells are summarized and plotted as a function of α-amanitin concentration in Fig. 3. For the 5.5S RNA (○) and for the 5S RNA (△) the α-amanitin concentration required for 50% inhibition is virtually the same as that required to inhibit by 50% purified RNA polymerase III from infected cells (com-

pare Fig. 1) or from other cell types (13, 14). Furthermore, the shapes of the inhibition curves are in all cases similar. Because of the increased resolution and relatively larger proportion of 5S RNA synthesized by these nuclei, the present data confirm and reinforce previous reports (14) which indicated that RNA polymerase III participated in the synthesis of cellular 5S RNA. These data also demonstrate that a class III RNA polymerase activity is responsible for the synthesis of viral 5.5S RNA in infected cells.

Previous reports (17, 18) demonstrated the sensitivity of adenovirus mRNA synthesis to α-amanitin and suggested the involvement of an RNA polymerase II activity. Since these experiments did not examine the exact sensitivity of mRNA synthesis to α-amanitin, this question has been reinvestigated. In the present experiments RNA was synthesized at various α-amanitin concentrations and at an ammonium sulfate concentration (0.06 M) optimal for endogenous RNA polymerase II (28). Total nuclear RNA was extracted and viral mRNA precursors (29) monitored by hybridization to viral DNA since the majority of the viral DNA sequences are believed to code for these RNAs. Since nuclei contain pools of viral RNAs, the hybridization conditions are those of RNA excess. However, the inhibitory action of the toxin on synthesis is apparent in this assay, since its effect is to reduce the specific activity of the RNA species that hybridize. The results (Fig. 3, ●) demonstrate that mRNA synthesis exhibits the same sensitivity to α-amanitin as does purified RNA polymerase II from infected KB cells (Fig. 1) and from other cell types (13–16). The residual activity at 0.4 μg/ml of α-amanitin probably represents hybridization of the products of the endogenous RNA polymerase III activity which accounts for 10–20% of the total activity at this salt concentration. Thus, the fraction of virus specific RNA (i.e., RNA that hybridizes to viral DNA) synthesized by RNA polymerase III is significantly lower than the fraction of the total RNA synthesized by RNA polymerase III in isolated nuclei (Fig. 1). In Fig. 1 total RNA synthesis (cellular plus viral) was monitored at a salt concentration (0.02 M) optimal for endogenous RNA polymerase III (28). Therefore, the experiments of Fig. 1 and Fig. 3 cannot be compared directly with respect to the relative activities of RNA polymerases II and III. Thus, the present data clearly demonstrate the function of an RNA polymerase II in transcription of the majority of the viral DNA sequences, although they do not exclude the possibility that RNA polymerase III is also involved to a limited extent in mRNA precursor synthesis.

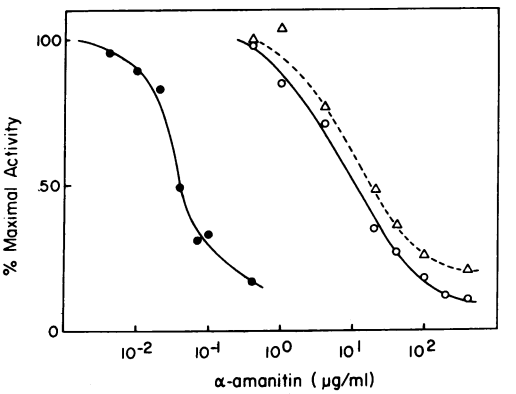

FIG. 3. Effect of α-amanitin concentration on the synthesis of viral mRNA precursors, 5.5S viral RNA, and host 5S RNA synthesis. Nuclei were incubated (●) in the presence of 0.06 M ammonium sulfate with various concentrations of α-amanitin; other conditions were as described in Fig. 2. The RNA synthesized was purified and hybridized to nitrocellulose filters containing 0.5 μg of adenovirus in 0.6 M NaCl, 0.06 M Na citrate, 0.2% Na dodecyl sulfate at 66° overnight (22). The 100% value represents 1557 cpm (2% of input) of RNase-resistant hybrid. The percentages of mRNA synthesized by nuclei, and annealed to viral DNA, are plotted as a function of α-amanitin concentration. The amounts of 5.5S RNA (○) and 5S RNA (△) synthesized *in vitro* in the presence of increasing α-amanitin concentrations (data from Fig. 2) are plotted as percentages of control values. The 100% control values were 5442 cpm for 5.5S RNA and 1580 cpm for 5S RNA. No backgrounds were subtracted, which may explain the apparent incomplete inhibition of 5.5S or 5S RNA synthesis.

DISCUSSION

The identification of the RNA polymerases involved in transcription of specific viral genes and an elucidation of how their activities are regulated is fundamental for understanding viral gene expression. Using isolated nuclei which faithfully transcribe at least some of those genes active *in vivo* and taking advantage of the distinct α-amanitin sensitivities of the nuclear RNA polymerases, we have identified two enzymes involved in adenovirus 2 transcription. The necessity and validity of this approach have previously been discussed (14) and nonspecific effects have been ruled out (ref. 14 and above).

Although nuclei prepared from cells late in infection are very active in RNA synthesis, no significant endogenous RNA polymerase I activity (less than 2% of the total activity) is demonstrable, since high α-amanitin concentrations completely inhibit endogenous RNA synthesis. This suggests that host RNA polymerase I is not involved in transcription of the adenovirus genome at this time in infection and supports other data (33, 34) indicating that host rRNA synthesis is grossly impaired in these cells. These observations must be reconciled with the observation that normal levels of RNA polymerase I activity can be demonstrated following solubilization (28). It is possible that viral induced or viral coded factors interfere with enzyme activity *in vivo* either at the chromatin or enzyme level. Similar observations have been made in picornavirus-infected cells where the activity of RNA polymerases I and II are inhibited both in intact cells and in isolated nuclei, despite the fact that normal levels of both enzymes can be detected after either solubilization or purification (35).

The suggestion that a class II RNA polymerase is involved in transcription of the bulk of the adenovirus genome (17, 18) is further reinforced by the more detailed analysis of the α-amanitin sensitivity of precursor mRNA synthesis (Fig. 3). Reanalysis of this question was necessary because of the existence of two RNA polymerases (II and III) with different α-amanitin sensitivities. The involvement of an RNA polymerase III activity in the synthesis of viral 5.5S RNA late in infection has been clearly demonstrated. Additional hybridization competition experiments indicate that species of viral RNA other than 5.5S RNA are also synthesized by an RNA polymerase III activity (unpublished observations). Furthermore, cellular 5S RNA and probably 4S RNA precursors continue to be transcribed in infected cell nuclei by an RNA polymerase III activity. Since the effects of α-amanitin on different RNA polymerases are specific, the

exact coincidence of the inhibition curve for the nuclear synthesis of a specific RNA with the inhibition curve for a specific RNA polymerase provides a stringent criterion for defining an enzyme function. This approach avoids the ambiguities present in attempts to define enzyme functions on the basis of effects of nonspecific agents (salts, metal ions, etc.), particularly in systems such as that described here where the endogenous RNA polymerase activities respond differently than do the purified enzymes.

It is interesting that two different RNA polymerases (II and III) are required to transcribe a genome as small as that of adenovirus 2. Although the presence of viral coded enzymes cannot be ruled out at the present time, the identical chromatographic properties (26, 28) and α-amanitin sensitivities of uninfected and infected cell enzymes suggest that the host enzymes are responsible for the transcription of the adenovirus genome. However, alterations in the activity and/or selectivity of the host class II and III enzymes in infected cells appears probable. This is suggested by the observation that the endogenous activities of these enzymes increase several fold during the course of infection (ref. 18 and unpublished observations), although the activity levels measured when the enzymes are solubilized and assayed with exogenous nonspecific templates remain about the same (28). Furthermore, in infected cells the enzymes appear to transcribe predominantly viral genes. Whether virus-coded or virus-induced factors mediate these changes by interacting with the enzymes or with the templates remains to be seen.

In the host cell, 5S RNA and tRNA have structural roles and are synthesized by an RNA polymerase III activity(ies) (14). Since the viral 5.5S RNA is synthesized by an analogous enzyme activity, and since it exists in large amounts in the infected cell (19), it may be that this RNA also has a structural role in viral-infected cells—perhaps in stimulating viral functions or in inhibiting host functions. However, despite our lack of knowledge about the function and importance of this RNA species, the 5.5S RNA system is an attractive model system for studying the transcription of a specific gene, for the RNA is small and does not appear to be processed (20). Moreover its complete sequence is known (36), and the enzyme that transcribes the corresponding viral gene has been identified (above) and is available in purified form. Finally the ability of isolated nuclei to reinitiate the synthesis of this RNA (20) suggests that any components necessary for specific transcription may be conveniently isolated and purified from this source.

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